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Original Paper

The Placental Secretome: Identifying Potential Cross-Talk Between Placenta and Islet β-Cells

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Key Words
G protein-coupled receptor • β-cell • Pregnancy • Placental ligand

Abstract
Background/Aims: Insulin-secreting islet β-cells adapt to the insulin resistance associated with pregnancy by increasing functional β-cell mass, but the placental signals involved in this process are not well defined. In the current study, we analysed expression of G-protein coupled receptor (GPCR) mRNAs in mouse islets and islet GPCR ligand mRNAs in placenta during pregnancy to generate an atlas of potential interactions between the placenta and β-cells to inform future functional studies of islet adaptive responses to pregnancy. ***Methods:*** Quantative RT-PCR arrays were used to measure mRNA expression levels of: (i) 342 GPCRs in islets from non-pregnant mice, and in islets isolated from mice on gestational days 12 and 18; (ii) 126 islet GPCR ligands in mouse placenta at gestational days 12 and 18. ***Results:*** At gestational day 12, a time of rapid expansion of the β-cell mass, 189 islet GPCR mRNAs were quantifiable, while 79 of the 126 known islet GPCR ligand mRNAs were detectable in placental extracts. Approximately half of the quantifiable placental GPCR ligand genes were of unknown function in β-cells. The expression of some islet GPCR and placental ligand mRNAs varied during pregnancy, with altered expression of both GPCR and ligand mRNAs by gestational day 18. ***Conclusion:*** The current study has revealed numerous potential routes for interaction between the placenta and islets, and offers an atlas to inform further functional studies of their roles in adaptive responses to pregnancy, and in the regulation of the β-cell mass.

Introduction

The increased insulin resistance that develops during normal pregnancy is compensated for by adaptations in islet β-cells, including higher rates of insulin secretion and increased β-cell mass [1-3]. Failure of the β-cells to adapt appropriately leads to glucose intolerance and the eventual development of overt gestational diabetes mellitus (GDM) [2-4]. In rodents, the β-cell expansion during gestation occurs over a relatively short and well-defined period [5, 6], offering a useful experimental model in which to study the regulation of functional β-cell mass. Islet β-cell adaptive responses occur after placentation and are rapidly reversed

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post-partum, consistent with placental signals playing a major role in informing β-cells of the gestational stage via circulating mediators. It is well established that the placental lactogens and prolactin play important roles in metabolic adaptations during pregnancy, but these cannot account for all of the β-cell adaptive responses because the time-course of elevations in their circulating levels does not match that of increases in β-cell mass [7-11]. The current study aimed to identify other placentally-derived mediators with the potential to influence β-cells to facilitate future functional studies into changes in the endocrine capacity of islets in pregnancy.

G-protein coupled receptors (GPCRs) are the most abundant mammalian family of cell surface receptors, and the human genome encodes 384 functional, non-odorant GPCRs [12]. We have demonstrated that human islets express 293 different GPCRs ("GPCRome") that enable β-cells and other islet endocrine cells to recognise and respond to a vast array of physiological signals [13], and islets from inbred C57 and outbred ICR (CD1) mice express mRNAs encoding 227 and 279 GPCRs respectively [14]. We have also recently studied cross-talk between mesenchymal stromal cells (MSCs) and islets using a mRNA library for known peptide or protein ligands for the islet GPCRome to identify MSC-derived secreted products ("secretome") with the potential to influence islet cell function via activation of GPCRs [15]. We here apply these approaches in pregnant mice to characterize the placental secretome and the islet GPCRome to identify novel placental ligands/islet GPCRs that may be involved in adaptive responses of islets to pregnancy.

Materials and Methods

Isolation of islets and placentas

Female CD1 mice (Charles River, UK, 8 weeks old) were mated and the subsequent presence of a vaginal plug was designated day 0 of pregnancy. Tissue samples were prepared at two stages of pregnancy: gestational day (gd) 12 in mid-pregnancy when β-cell mass expansion is maximal; and gd18 in late pregnancy when the β-cell mass is no longer expanding [6]. Placenta were removed and extracted immediately while islets were isolated from whole pancreas by collagenase digestion, as described previously [15].

Measurement of gene expression by quantitative RT-PCR

Quantative RT-PCR (qRT-PCR) was used to measure the mRNA expression levels of: (i) 342 GPCRs in islets isolated from non-pregnant mice, and from islets isolated at gds12 and 18; (ii) 126 islet GPCR ligands in mouse placenta at gd12 and gd18, using groups of six mice at each time point. Total RNA was isolated using RNeasy Mini kits (Qiagen), and RNA quality was assessed using the ND1000 spectrophotometer (Nanodrop). RNA samples with A_{260}/A_{280} ratios between 1.8 and 2.2 were considered sufficiently pure for experimental use and were converted into cDNAs using the High Capacity Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. cDNA samples were interrogated by quantitative RT-PCR (qRT-PCR) using a LC480 Light Cycler (Roche). Bioinformatically validated QuantiTect primer assays (Qiagen) were used for the expression analysis using a QuantiTect SYBR Green PCR Kit (Qiagen). A full list of primers is shown (for all online suppl. material, see www.karger.com/doi/10.1159/000487357) in Suppl. Tables 1 and 2. Reactions producing specific DNA products (homogeneous melting curves) were further analysed by 2% agarose gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 7.6) to confirm that sizes of generated products matched the primer specifications. Relative expression of target genes was determined by the $2^{-\Delta\Delta Ct}$ method using a Ct value of 20 for all screening and quantification, and GAPDH as a reference gene [16].

Statistical analysis

Differences between two groups were analysed by unpaired Student's t-test with Welch's correction. For comparisons between more than two groups, one-way analysis of variance (ANOVA) with Tukey's (equal variances) or Dunnett's T3 (unequal variances) post hoc tests were used. Group variances were compared using Bartlett's test for equal variances. Data were processed using Prism (GraphPad) or SPSS Statistics 23 (IBM) software and expressed as means±SEM.

Results

Islet GPCRome during pregnancy

We compared GPCR mRNA expression levels in islets isolated from non-pregnant mice with islets isolated from pregnant mice on gd12 and gd18. In all islet extracts the most abundantly expressed GPCR mRNAs were GPR56, GPR158, FFAR1, GIPR, GALR1 and GALR3, with expression levels an order of magnitude greater than those of the other GPCR mRNAs, consistent with our previous report [14]. There were no significant differences in the expression of these GPCR mRNAs between non-pregnant, gd12 and gd18 islets so they were omitted from the expression plots (Fig. 1, 2) for ease of scaling, although the qRT-PCR data for these GPCR mRNAs are included in (see online suppl. material) Suppl. Tables 3 and 4. When compared to non-pregnant expression levels 71 GPCR mRNAs were differentially expressed on gd12, as shown in Fig. 1 and (see online suppl. material) Suppl. Table 3, with 26 of these being upregulated (Fig. 1, red symbols). By day 18 of gestation 66 GPCR mRNAs were differentially expressed compared to non-pregnant islets, as shown in Fig. 2 and (see online suppl. material) Suppl. Table 4, with 35 of these being upregulated (Fig. 2, red symbols). Figures 1 and 2 also show the changes in GPCR mRNA expression between gd12 and gd18, respectively, with upregulated mRNAs being denoted by triangles, down-regulated mRNAs being denoted by squares and mRNAs whose expression did not change being shown by solid circles

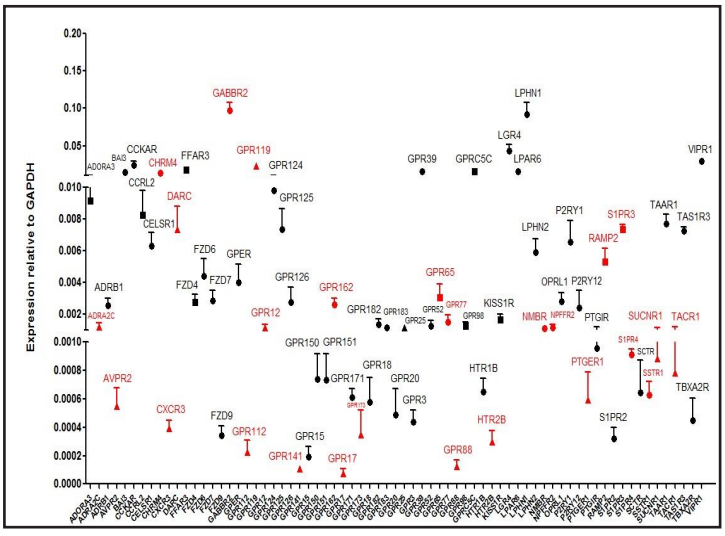

Fig. 1. Islet GPCRome on gestational day 12. Red markers: islet GPCR mRNAs upregulated on gd12 vs non-pregnant islets; black markers: mRNAs downregulated on gd12 vs non-pregnant islets. Solid triangles show mRNAs upregulated in gd12 vs gd18 islets; solid circles show mRNAs not differentially expressed on gd12 vs gd18 islets; solid squares show mRNAs downregulated in gd12 vs gd18 islets. Values are expressed as means ± SEM, n = 4-6; all mRNAs p<0.05. Full data set and abbreviations are shown (see online suppl. material) in the Suppl. Table 3.

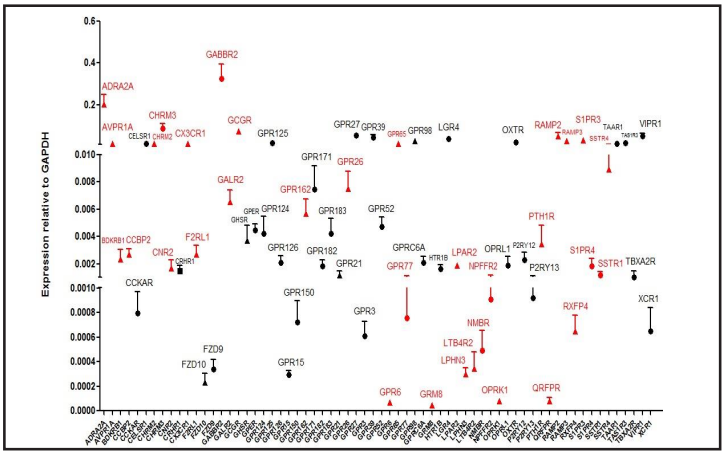

Fig. 2. Islet GPCRome on gestational day 18. Red markers: islet GPCR mRNAs upregulated on gd18 vs non-pregnant islets; black markers: mRNAs downregulated on gd18 vs non-pregnant islets. Solid triangles show mRNAs upregulated in gd18 vs gd12 islets; solid circles show mRNAs not differentially expressed on gd18 vs gd12 islets; solid squares show mRNAs downregulated in gd18 vs gd12 islets. Values are expressed as means ± SEM, n = 4-6; all mRNAs p<0.05. The full data set and abbreviations are shown (see online suppl. material) in the Suppl. Table 4.

Mouse placental secretome

On gd12, a time of rapid β-cell expansion [6], 79 out of 126 known islet GPCR ligand mRNAs were expressed at detectable levels in mouse placental extracts, as shown in Fig. 3 and (see online suppl. material) Suppl. Table 5. Analysis of the expression of the same mRNA species in placental extracts at gd18, when β-cell expansion has ceased [6], indicated expression of 62 GPCR ligand mRNAs (Fig. 4, see online suppl. material, Suppl. Table 6). Comparison of the data sets generated from placentas retrieved at gd12 and gd18 revealed a number of time-dependent changes in the placental secretome. Thus, expression of 35 islet GPCR ligand mRNAs was upregulated (Fig. 3, red symbols), while the expression of 17 mRNAs was downregulated (Fig. 3, blue symbols). Conversely, comparing expression at gd18 with that at gd12 indicated that expression of 26 islet GPCR ligand mRNAs was upregulated (Fig. 4, red symbols), while the expression of 9 mRNAs was downregulated (Fig. 4, blue symbols). In addition, expression of 27 placental islet GPCR ligand mRNAs did not change between gd12 and gd18 (Figs. 3 and 4, black symbols).

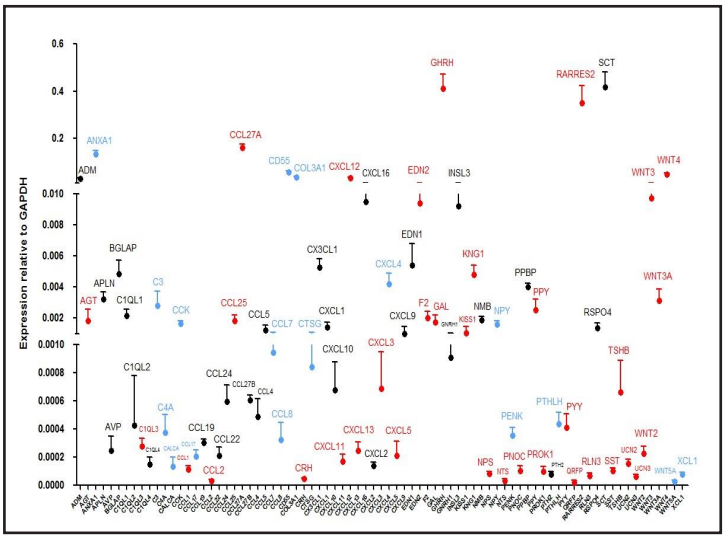

Fig. 3. Placental GPCR ligand secretome on gestational day 12. Red markers: mRNAs upregulated on gd12 vs gd18 expression levels; black markers: mRNAs not differentially expressed on gd12 vs gd18 expression levels; blue markers: mRNAs downregulated on gd12 vs gd18 expression levels. Values are expressed as means ± SEM, n = 4-6; red and blue $p<0.05$. Full data set and abbreviations are shown (see online suppl. material) in Suppl. Table 3.

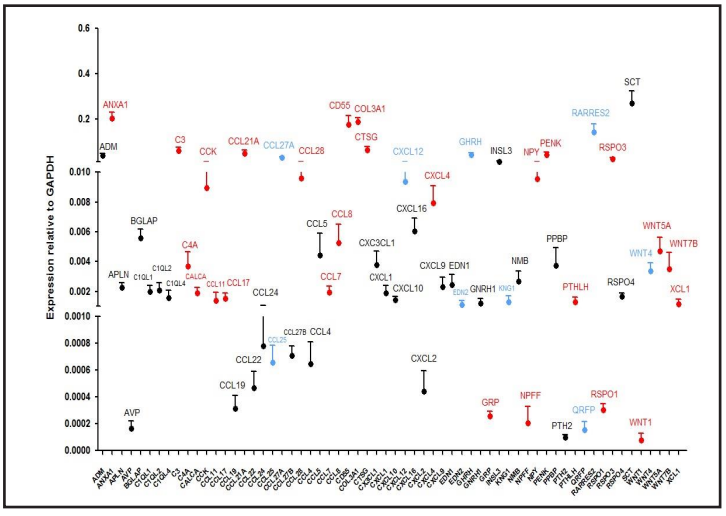

Fig. 4. Placental GPCR ligand secretome on gestational day 18. Red markers: mRNAs upregulated on gd18 vs gd12 expression levels; black markers: mRNAs not differentially expressed on gd18 vs gd12 expression levels; blue markers: mRNAs downregulated on gd18 vs gd12 expression levels. Values are expressed as means ± SEM, n = 4-6; red and blue $p<0.05$. Full data set and abbreviations are shown (see online suppl. material) in Suppl. Table 6.

Cross talk between placenta and islets

Placenta-derived islet GPCR ligands that have reported effects on insulin secretion and/or β-cell proliferation were identified by manual searches on PubMed. As shown in Fig. 5, over 50% of the placental ligands that were identified in our secretome analyses were of unknown function in islets. Of the remaining ligands whose effects on β-cells or islets have been reported, over 50%

had stimulatory effects, around 20% had inhibitory effects, and the remainder were reported to have both stimulatory and inhibitory effects on β-cell function (Fig. 5). The full data set is shown (see online suppl. material) in Suppl. Table 7.

To focus on placental ligands and their cognate islet GPCRs that are likely to be involved in adaptive responses to pregnancy we identified islet GPCR mRNAs that showed increased expression at gd12 versus non-pregnant and/or gd18 expression, and for which ligands were expressed in the placenta at gd12. Fig. 6 shows eight GPCR mRNAs whose expression was upregulated in islets on gd12 and whose ligand mRNAs were also expressed in the placenta retrieved from mice at gd12. Of these eight islet GPCRs, ligands for three receptors (*Crh/Ucn2, F2, Sst*) were upregulated, the ligand for one receptor (*Cck*) was downregulated, and the ligands for four receptors (*Avp, Nmd, Adm*) did not change, when comparing the ligand mRNA expression in the placenta at gd12 versus gd18.

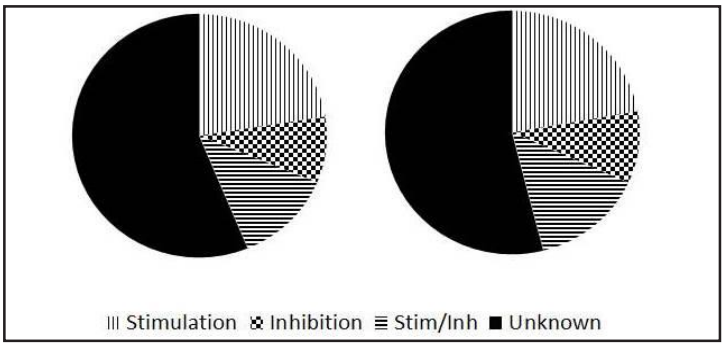

Fig. 5. Reported effects of placental GPCR ligand secretome on β-cell function. The reported effects on β-cell function of placental GPCR ligands expressed on gd12 (A) or gd18 (B) are either unknown (Unknown, solid black: A=56%, B=54%), stimulatory (Stimulation, vertical bars: A=22%, B=22%), inhibitory (Inhibition, cross hatch: A=9%, B=10%) or both stimulatory and inhibitory depending on the species and experimental conditions (Stim/Inh, horizontal bars: A=13%, B=14%). The full data set is shown (see online suppl. material) in Suppl. Table 7.

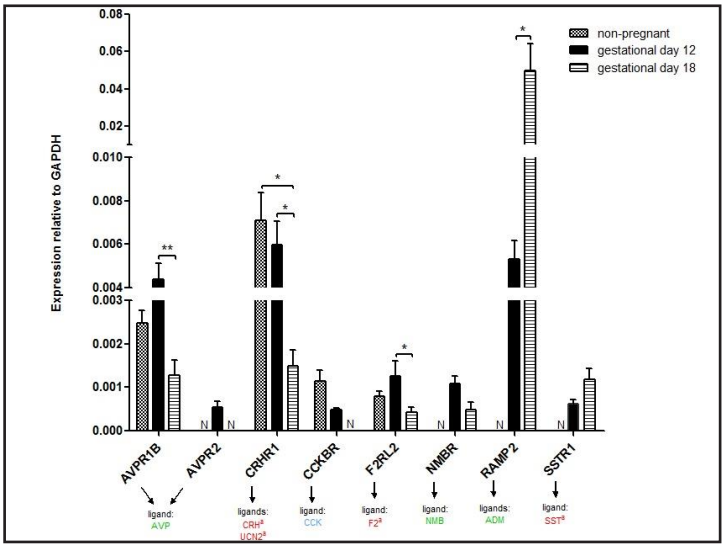

Fig. 6. Mouse islet GPCR mRNAs and placental ligand mRNAs at gestational day 12. Bars show mRNA expression levels of 8 islet GPCRs which were upregulated on gd12 (solid bars) compared to gd18 (cross-hatched bars). Expression levels in islets isolated from non-pregnant animals is shown by the stippled bars, where detectable. Text shows expression status of placental ligand mRNAs at gd12. Red text: ligand mRNAs upregulated on gd12 vs gd18; blue text: ligand mRNA downregulated on gd12 vs gd18; green text: ligand mRNAs not deferentially expressed on gd12 vs gd18; N: not detectable/not quantifiable expression; [a]: ligand mRNAs expressed in the placenta on gd12 but not expressed on gd18. Values are expressed as means ± SEM, n = 4-6; one-way ANOVA test * p<0.05, ** p<0.01.

Discussion

Under normal circumstances in adults β-cells are essentially non-proliferative and the functional β-cell mass is static. However, increases in peripheral insulin resistance induce a compensatory increase in β-cell mass [1-3]. Failure of the β-cell mass to adapt appropriately to pregnancy or obesity results in β-cell insufficiency, leading to glucose intolerance and the eventual development of overt GDM or type 2 diabetes mellitus (T2DM),

respectively. Understanding the β-cell adaptive responses may inform therapeutic interventions in women at risk of developing GDM, and also in the much larger population at risk of developing T2DM. In addition, understanding how the β-cell functional mass is upregulated during pregnancy may have important repercussions for the generation *in vitro* of β-cell graft material for the transplantation therapy of Type 1 diabetes. The aim of this study was therefore to produce an atlas of the islet GPCRome and placental peptide ligand secretome during pregnancy to identify novel placentally-expressed ligands with the potential to influence β-cell function.

Most previous studies on placental regulation of islet function have focused on ligands whose receptors are coupled via the JAK/STAT pathways, such as the placental lactogens, or whose receptors have intrinsic tyrosine kinase activity, such as the growth factor receptor families. We chose to investigate GPCRs because this is the most abundant family of cell surface receptors, and because we have previously demonstrated that β-cells express hundreds of GPCRs, many of unknown function [13, 14]. Some GPCRs have been implicated previously in β-cell adaptive response to pregnancy. For example, serotonin mediates the pro-proliferative and insulinotropic action of lactogenic hormones in maternal β-cells through activation of its Gαs protein-coupled GPCR, Htr2b, during mid-gestation in mice [8].

We have identified considerable potential for cross-talk between placenta and islets via secreted placental ligands that could interact with islet GPCRs. Not all of the ligands whose mRNAs were identified in the mouse placental secretome will be involved in regulating maternal responses to pregnancy. Some, such as the chemokines, are likely to be involved in local maintenance of placental function [17] while others, such as placental growth hormones, may be involved in the regulation of fetal development [18]. However, the expression of so many ligands with the potential to influence islet function via GPCR signalling, and the knowledge that lactogenic hormones cannot alone account for all β-cell adaptive responses to pregnancy [7] are consistent with the notion that some of these ligands may be involved in placenta/β-cell cross-talk.

The current study aimed to produce an atlas of islet GPCRs/placenta-derived ligands to guide future functional studies. However, given the large numbers of GPCR mRNAs expressed by the maternal islets, and the large range of potential ligand mRNAs expressed by the placenta, identifying functional GPCR/ligand combinations requires the application of some selection criteria. One strategy is to compare the patterns of expression with those of the lactogenic hormones known to be involved in β-cell adaptive responses to pregnancy [7, 10]. Thus, expression of the islet GPCR should increase when the β-cell mass is actively expanding (gd12) and may be down-regulated when the β-cell mass expansion is complete (gd18). Similarly, the placental ligand(s) should be highly expressed during the β-cell expansion phase, and may fall towards the end of gestation. Our data identified a number of islet GPCR mRNAs upregulated on gd12 whose ligand mRNAs were expressed by the placenta at gd12, so some of these may be useful candidates for further functional studies. For example, corticotrophin releasing hormone (CRH) is an interesting candidate. We have shown that both islet *Crhr1* and placental *Crh* were upregulated on gd12 compared to gd18, and previous *in vitro* studies have demonstrated that CRH enhances insulin secretion [19, 20] and β-cell proliferation [20]. Together, these observations are consistent with a potential role for CRH in signaling from the placenta to the islet during pregnancy.

In conclusion, the mechanisms underlying the well-documented β-cell adaptations to pregnancy are not fully understood, although the failure of these mechanisms is implicated in the development of GDM. The current study has revealed numerous potential routes for interaction between the placenta and β-cells, and offer an atlas to guide further functional studies of their roles in islet adaptive responses to pregnancy.

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Disclosure Statement

The authors have nothing to disclose.

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